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### Preeclampsia is Associated with Lower Percentages of Regulatory T Cells in Maternal Blood

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# Preeclampsia is Associated with Lower Percentages of Regulatory T Cells in Maternal Blood

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**Objective:** Immunological mechanisms are involved in the pathophysiology of preeclampsia. During pregnancy there is an increase in regulatory T (Treg) cells, which has an important role in regulating tolerance to the immunologically distinct fetus. We hypothesised that percentages of Treg cells are decreased in preeclamptic patients. **Methods:** Peripheral blood was obtained from 26 healthy pregnant controls and 18 preeclamptic patients. Treg cells were measured using flow-cytometry. **Results:** Women with pregnancies complicated by preeclampsia had significantly lower percentages of CD4<sup>+</sup>FOXP3<sup>+</sup> Treg cells. **Conclusion:** We conclude that a deficiency of regulatory T cells may play a role in the pathophysiology of preeclampsia.

**Keywords** Case-control study, Cell biology, Inflammation, Preeclampsia, Pregnancy.

## INTRODUCTION

Despite great advances in pre- and perinatal care it is estimated that worldwide more than 50,000 women die from preeclampsia per year (1). In developed countries preeclampsia is responsible for 15–20% of maternal mortality (2). Preeclampsia can manifest as a maternal syndrome (hypertension, proteinuria

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and oedema) and/or a fetal syndrome (fetal and placental growth restriction, reduced amniotic fluid and abnormal oxygenation) (3). The etiology and pathogenesis of preeclampsia are not fully known (1,2,4,5). However, abundant evidence suggests that immunological factors are crucial for the development of this disease (2,6,7).

For several years the Th1-Th2 paradigm has been used to explain the maternal tolerance towards the fetus. During normal pregnancy the maternal immune system shifts to a pre-dominant Th2 response, which is thought to be important in tolerance to the immunological distinct fetus (7,8). The shift to a pre-dominant Th2 response probably does not occur or is reversed at some point during gestation in patients with pregnancies complicated by preeclampsia (6,7,9). Th1 dominance develops together with an increased inflammatory response towards the fetus (10). However, this explanation has been questioned in light of recent research, and it has become clear that additional regulatory mechanisms play a role in the fetal-maternal tolerance (10,11). Regulatory T (Treg) cells are regarded as having an important role in regulating the immune response and tolerance to the fetus (8,10,12,13). Normal pregnancy is associated with an elevation of Treg cells in maternal blood and decidua (8,14,15). As Treg cells can also suppress NK cells and NKT cells, in terms of proliferation and cytokine production, they might suppress type-1 responses in normal pregnancy by this route (10).

Treg cells are a subpopulation of CD4<sup>+</sup>CD25<sup>+</sup> lymphocytes specifically characterized by the lineage specific transcription factor Forkhead Box P3 (FOXP3) (13,16–19). It has been shown that Treg cell function requires the expression of FOXP3 (17,19,20). Treg cells are furthermore characterised based on expression of several surface markers; besides CD25, these include GITR (glucocorticoid-induced TNFR) and CTLA-4 (cytotoxic T-lymphocyte associate protein 4, CD152), whose expression on Treg cells is dependent on FOXP3 (13,21).

Several studies have been published describing an association between a decreased level of Treg cells in peripheral blood and preeclampsia, however the methods and results are not consistent (22–26). Furthermore, most of these studies used CD25 as a Treg cell marker. However surface markers such as CD25 are also dynamically expressed on the surface of newly activated CD4<sup>+</sup> T-helper cells. Using the intracellular marker Foxp3 is therefore superior and more accurate. Interestingly, some studies have shown an association between preeclampsia and changed levels of T cell subpopulations in umbilical cord blood, showing an alteration in the immunological parameters of the newborn (27,28).

The aim of this study was to investigate any association between pregnancies complicated by preeclampsia and levels of Treg cells in peripheral maternal blood and umbilical cord blood. We therefore analysed peripheral blood and

umbilical cord blood samples from women whose pregnancies were complicated by preeclampsia and samples from women with a normal pregnancy.

## MATERIAL AND METHODS

### Patient Details

This study was approved by the Medical Ethical Committee at the University Medical Center Groningen (UMCG). Women were recruited from the department of Obstetrics and Gynaecology of UMCG, the Netherlands. All women gave written informed consent. In total, 18 women with pregnancies complicated by preeclampsia and 26 healthy pregnant controls were enrolled in this study. The healthy controls were divided in two groups; term (control group I) and preterm (control group II). Control group I consisted of eighteen healthy pregnant women at term, opting for elective Caesarean section. Since almost all the women with preeclampsia delivered preterm, control group II (peripheral blood only) consisted of 8 women during normal pregnancy with a gestational age matched for the preterm preeclamptic group to determine the possible influence of pregnancy length on the levels of Treg cells.

Preeclampsia was defined as gestational hypertension (systolic blood pressure  $\geq 140$  mmHg and/or a diastolic blood pressure  $\geq 90$  mmHg after 20th week of gestation) combined with significant proteinuria ( $\geq 300$  mg/24 hour). Women with other disorders of the immune system or using (other) immune suppressing medication were excluded from this study.

### Samples

Peripheral blood and umbilical cord blood samples were collected in Lithium-heparin tubes. All peripheral blood samples were obtained well before the onset of labor or caesarean section. Due to logistic circumstances and transfers of the patients it was only possible to collect an umbilical cord blood sample of 8 preeclamptic patients and 16 healthy controls.

### Analysis of Regulatory T Cells by Flow-Cytometry

First, the samples were divided into a tube for intracellular staining and one for extracellular staining, as the protocols are different. Staining for FOXP3 (intracellular staining). In each test tube 200  $\mu$ l whole blood was pipetted, after washing the blood with PBS and centrifugation, the supernatant was discarded. The cells were fixed and permeabilised using a fixation-permeabilization buffer (eBioscience, USA), and incubated for 30 minutes at 4°C. The cells were then washed once with PBS solution and twice with permeabilization buffer (eBioscience, USA). This was followed by incubation for 15 minutes at 4°C after addition of normal rat serum to reduce aspecific

binding. The cells were then incubated for 30 minutes at 4°C with PerCP-, APC- and PE- labelled antibodies against CD4, CD25 and FOXP3, respectively, or appropriate isotype controls. The PE anti-human FoxP3 staining set was obtained from eBioscience, USA, all the other antibodies were purchased from BD Pharmingen, USA.

This was followed by washing twice with permeabilization buffer, after which the cells were resuspended in 400 µl FACS buffer, consisting of PBS, 1% BSA and 0,1% NaN<sub>3</sub>. Samples were analysed on the FACSCalibur™ within 4 hours.

Staining for CTLA-4 and GITR (cell surface staining): First the antibodies (CD4-Per-CP, CD25-APC, CTLA4-PE and GITR-FITC) or appropriate isotype controls were pipetted in FACS tubes. Then 200 µl whole blood was added to each tube. After the cells were incubated for 15 minutes at 20°C, red blood cells were lysed with IQ-lyse (IQ products, Groningen, the Netherlands) and stored for 10 minutes in the dark at 20°C. Hereafter demineralised water was added and the tubes were stored again for 10 minutes at 20°C. Then cells were resuspended in 400 µl FACS buffer. Samples were analysed on the FACSCalibur™ within 4 hours.

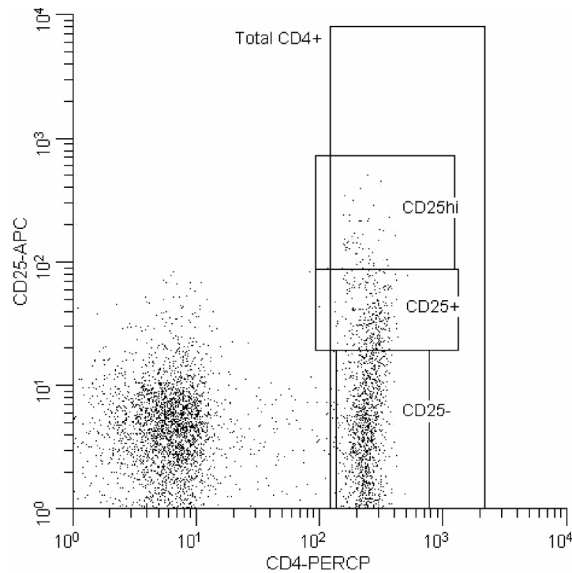
For each sample 50,000 cells were acquired for analysis. Cells were measured using a BD FACSCalibur™ with CELLQuest™ software. For the acquisition a real time gate was placed around the lymphocytes based on their forward scatter/side scatter profile, avoiding other cell populations. Subsequently the cells were analysed using Winlist 6.0. Cell populations were identified based on histograms and dot plots. A CD25<sup>high</sup> subpopulation was identified within the CD25<sup>+</sup> population (see Figure 1).

## Statistics

For the comparison of the obstetric and maternal characteristics between the cases and controls the Mann-Whitney U-test and Fisher's exact test were used. SPSS software was used for this, *p*-values <0.05 were considered to be significant. Graphpad Prism was used for designing of the graphs. For the analysis of the frequencies of cells that stained positively with different antibodies the Mann-Whitney test was used.

## RESULTS

The frequency of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells in peripheral maternal blood was not significantly decreased in women with preeclampsia. However, the frequency of CD4<sup>+</sup>FOXP3<sup>+</sup> T cells in peripheral blood was significantly lower in women with preeclampsia than in healthy pregnant controls (*p* < 0.01), for the frequencies of T cells that stained positively with the superficial markers we found no significant differences (Table 1). Except for the significantly



**Figure 1:** Expression of CD25 in CD4<sup>+</sup> cells. The gates are set with help of isotype controls.

**Table 1:** Comparison of percentages of T cells that stained positively with different antibodies in peripheral blood between the cases and controls (levels as percentage of CD4<sup>+</sup> cells).

	Healthy controls (n = 26)	Preeclamptic women (n = 18)	Significance
Maternal blood (mean ± SD)			
CD4 <sup>+</sup> FOXP3 <sup>+</sup>	8.8 ± 5.4	4.5 ± 2.8	S
CD4 <sup>+</sup> CD25 <sup>+</sup>	5.4 ± 2.8	6.0 ± 2.1	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> FOXP3 <sup>+</sup>	1.5 ± 1.2	0.92 ± 0.95	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> CTLA-4 <sup>+</sup>	1.1 ± 0.77	0.95 ± 0.46	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> GITR <sup>+</sup>	0.20 ± 0.34	0.21 ± 0.19	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> CTLA-4 <sup>+</sup> GITR <sup>+</sup>	0.25 ± 0.25	0.23 ± 0.22	NS

SD = standard deviation; NS = not significant; S = significant;  $p < 0.01$ .

increased percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $p < 0.01$ ), the frequencies of T cells that stained positively with both the extracellular and intracellular markers in umbilical cord blood were comparable in both groups (Table 2).

Delivery was vaginal in 3 cases of preeclampsia, all other women underwent a caesarean section. Significant differences were found between the healthy pregnant controls and the preeclamptic women for parity, gestational age, infant weight and usage of corticosteroids. These differences were expected based on known associations with preeclampsia. A significant difference between the preeclamptic women and the gestational age matched

**Table 2:** Comparison of percentages of T cells that stained positively with different antibodies in umbilical cord blood between the cases and controls (levels as percentage of CD4+ cells).

	Healthy controls (n = 16)	Preeclamptic women (n = 8)	Significance
Umbilical cord blood (mean $\pm$ SD)			
CD4 <sup>+</sup> FOXP3 <sup>+</sup>	7.2 $\pm$ 3.3	7.9 $\pm$ 4.8	NS
CD4 <sup>+</sup> CD25 <sup>+</sup>	7.0 $\pm$ 1.7	9.4 $\pm$ 1.8	S
CD4 <sup>+</sup> CD25 <sup>high</sup> FOXP3 <sup>+</sup>	1.9 $\pm$ 1.1	1.5 $\pm$ 2.0	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> CTLA-4 <sup>+</sup>	1.9 $\pm$ 1.0	2.4 $\pm$ 1.7	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> GITR <sup>+</sup>	0.29 $\pm$ 0.36	0.25 $\pm$ 0.25	NS
CD4 <sup>+</sup> CD25 <sup>high</sup> CTLA-4 <sup>+</sup> GITR <sup>+</sup>	0.46 $\pm$ 0.45	0.38 $\pm$ 0.36	NS

SD = standard deviation; NS = not significant; S = significant;  $p < 0.01$ .

**Table 3:** Comparison of the obstetric and maternal characteristics between the cases and controls.

	Healthy controls (n = 18)	Preeclamptic women (n = 18)	Significance I	Controls II (n = 8)	Significance II
Maternal age (years) (mean $\pm$ SD)	32.2 $\pm$ 4.3	30.3 $\pm$ 6.1	NS <sup>†</sup>	32.6 $\pm$ 6.0	NS <sup>†</sup>
Parity					
primiparous	6 (33.3)	15 (83.3)	S <sup>†</sup>	6 (75.0)	NS <sup>‡</sup>
multiparous	12 (66.7)	3 (16.7)		2 (25.0)	
Gestational age (days) (median, range)	275.5	225.0	S <sup>†</sup>	236.0	NS <sup>†</sup>
	253–297	190–284		196–255	
Infant weight (grams) (mean, range)	3676.9	1961.2	S <sup>†</sup>		
	2420–4540	750–4130			
Smoking	5 (27.8)	1 (5.6)	NS <sup>‡</sup>	1 (12.5)	NS <sup>‡</sup>
Corticosteroids	0 (0.0)	12 (66.7)	S <sup>†</sup>	0 (0.0)	S <sup>†</sup>

The numbers in parentheses represent the percentage of women in each group.

SD = standard deviation; NS = not significant; S = significant;  $p < 0.05$ .

Significance I = significance between healthy controls and preeclamptic women.

Significance II = significance between preeclamptic women and gestational age matched controls (controls II).

<sup>†</sup>Mann-Whitney U-Test.

<sup>‡</sup>Fisher's exact test.

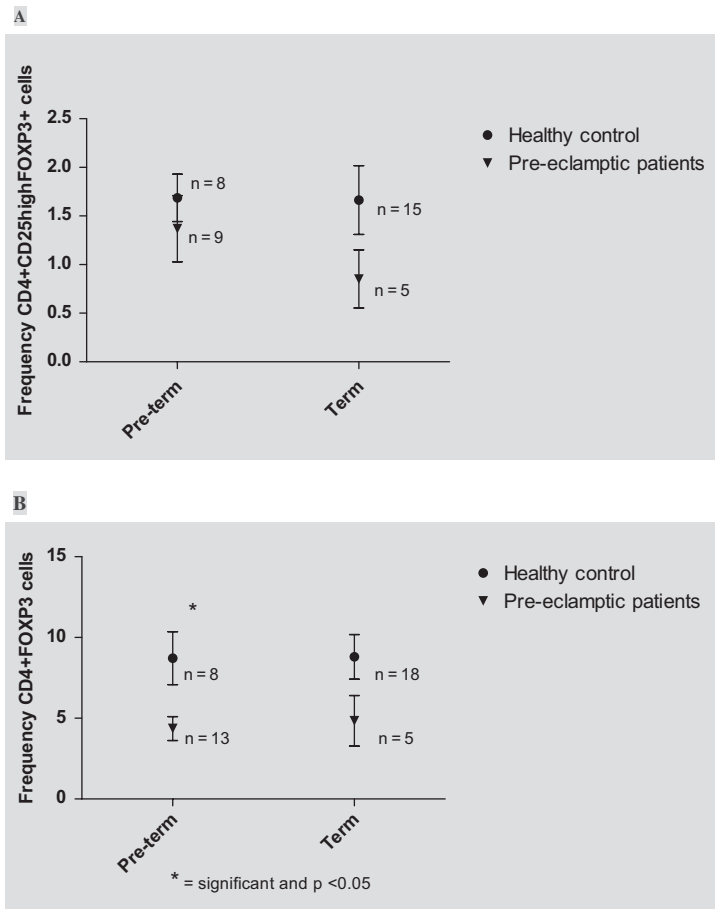
controls was found for the usage of corticosteroids (table 3), which was also expected.

According to the time of onset, preeclampsia can be divided in two sub-groups, namely, the early and the late onset forms. To investigate if there was a relationship between percentages of Treg cells and gestational age in women with and without preeclampsia, we divided all the subjects into pre-term



(<259 days of gestational age) and term ( $\geq 259$  days of gestational age) group (Figure 2). No significant differences were found for the CD4<sup>+</sup>CD25<sup>high</sup>FOXP3 staining (Figure 2A). However there was a decreased level of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3 cells in preeclamptic patients (Figure 2A). The pre-term group of pregnancies complicated by preeclampsia showed a significantly lower percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> cells in peripheral blood compared to the healthy pregnant controls (Figure 2B). At term this percentage was also lower but not significantly so. Between pre-term and term pregnancies complicated by preeclampsia no significant difference was found in the percentage of CD4<sup>+</sup>FOXP3<sup>+</sup>Treg cells (Figure 2B).

Furthermore, our data shows a small increase in the percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> cells in preeclamptic women using corticosteroids, compared to



**Figure 2:** Comparison of the frequency of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> cells (A) and CD4<sup>+</sup>FOXP3<sup>+</sup> cells (B) in peripheral maternal blood (levels as percentage of CD4<sup>+</sup> cells) in healthy pregnant controls and preeclamptic patients.

the non corticosteroids using preeclamptic patients. This difference was non-significant (results not shown). There was no difference in the level of CD4<sup>+</sup>FOXP3<sup>+</sup> cells in the blood of the women with a vaginal delivery compared with the rest of the respective group.

## DISCUSSION

In this study we did not find significantly decreased frequencies of CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> Treg cells in peripheral maternal blood of preeclamptic patients. There was however, a clear trend towards decreased frequencies.

Recently, two studies reported that preeclampsia is not associated with changes in the percentages of CD4<sup>+</sup>CD25<sup>+</sup> cells in peripheral blood (25,26). Both of these studies used the surface marker CD25 only for the detection of Treg cells. Our results confirm the similar frequencies of CD4<sup>+</sup>CD25<sup>+</sup> cells between the control and preeclamptic group. However, surface markers as for example CD25, CTLA-4, and GITR are also dynamically expressed on the surface of newly activated CD4<sup>+</sup> T-helper cells (29). Therefore it is difficult to discriminate Treg cells from newly activated T-helper cells based on surface phenotype alone, especially in pregnancy where there is normal activation of CD4<sup>+</sup> cells in response to paternal allo-antigens expressed by the fetus.

Sasaki et al. reported that levels of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> are decreased in peripheral blood and placental tissue of women with preeclampsia (24). Although we did not find significantly decreased levels of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells, we did find a negative trend in levels of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells in peripheral blood of pregnancies complicated by preeclampsia. Furthermore, our study differs in methodology and we measured FOXP3 in a larger number of preeclamptic patients.

It is known that not only CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells but also CD4<sup>+</sup>CD25<sup>-</sup>FOXP3<sup>+</sup> cells have a regulatory function, several papers have shown that Treg cells also can be found in the CD25<sup>-</sup>Foxp3<sup>+</sup> population (30,31). Therefore the CD4<sup>+</sup>FOXP3<sup>+</sup> population could be regarded as a more satisfactory and complete marker for Treg cells.

In this study we found significantly lower percentages of CD4<sup>+</sup> FOXP3<sup>+</sup> Treg cells in peripheral blood of women with pregnancies complicated by preeclampsia compared to healthy pregnant controls. In contrast to the lower percentages of Treg cells in the peripheral blood, no significant differences of CD4<sup>+</sup>FOXP3<sup>+</sup> Treg cells were found in umbilical cord blood.

Some studies suggest that during normal pregnancy the maternal immunological shift to a Th2 response takes place to promote tolerance to the fetus (7,8). It is thought that FOXP3<sup>+</sup> Treg cells have an important role in regulating the immune response and tolerance to the fetus, doing this by suppressing the maternal immune reaction towards the fetus (8,10,12,13). Treg cells can mediate their suppressive function through the action of cytokines and via cell

contact dependent mechanisms (32). These observations together with the finding that during normal pregnancy the percentages of Treg cells are increased (8,14), suggest that the lower percentage of FOXP3<sup>+</sup> Treg cells we found in the peripheral blood from women with preeclampsia may reflect impaired tolerance to the fetus in preeclampsia.

Women with preeclampsia were significantly more often treated with corticosteroids. Corticosteroids are used to prevent respiratory distress syndrome in premature infants. Several studies have found an increase in FOXP3 expression and an increase in the percentages of Treg cells as a result of the administration of corticosteroids (33,34). The comparison between the groups and the exposure to steroids would be much better if the possible steroid effect was avoided. However, because of ethical reasons and logistic circumstances it was not possible to measure these levels before administration of corticosteroids. The increase in the percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> cells in preeclamptic women using corticosteroids, compared to the non corticosteroids using preeclamptic patients suggests that percentages of Treg cells would be lower in pregnancies complicated by preeclampsia if no corticosteroids had been used.

The gestational age of the pregnancies complicated by preeclampsia was significantly lower from the gestational age of the healthy controls. As several studies showed changing levels of regulatory T cells during pregnancy (14,15,35), a healthy control group with a comparable gestational age to the preeclamptic pregnancies was included. However, percentages of Treg cells were comparable in both control groups.

When comparing the percentages of Treg cells between preeclamptic women and gestational age matched controls we found a lower percentage of Treg cells in the peripheral blood of women with a pregnancy complicated by preeclampsia. This confirms that Treg cell percentages are lower in pregnancies complicated by preeclampsia.

Mean Fluorescence Intensity (MFI) describes on a relative scale the amount of fluorescently labelled antibody which is attached to the antigen. Recently, using MFI, it was shown that in a mouse model, Foxp3 regulates Treg cell development and function in a dose-dependent, non-binary manner, and that decreased Foxp3 expression causes immune disease by subverting the suppressive function of Treg cells and converting Treg cells into effector cells (36). Our data on MFI measurements shows no significant differences between the preeclampsia group and gestational age matched controls (results not shown). This indicates that the percentage of FOXP3 positive cells as a fraction of the Treg cells is not different in pregnancies complicated by preeclampsia.

Our finding that frequencies of CD4<sup>+</sup>CD25<sup>+</sup> cells in peripheral blood were similar in the preeclamptic and healthy pregnant women suggests that systemic activation of T cells is not the main dysfunctional mechanism in preeclampsia. This is confirmed by comparable GITR and CTLA-4 expression

we found in both groups, as it is known that CTLA-4 and GITR are up-regulated when T cells are activated (13,21,37,38). Furthermore, GITR and CTLA-4 are involved in the function of Treg cells, the comparable relative expression of CTLA-4 and GITR we found in Treg cells shows that there are no large differences in percentages of functional Treg cells. The functional characteristics of the Treg cells could be further investigated with suppression assays on maternal blood of patients with preeclampsia.

In umbilical cord blood from pregnancies complicated by preeclampsia we found significantly higher percentages of CD4<sup>+</sup>CD25<sup>+</sup> cells than in umbilical cord blood from gestational age matched controls. This finding remains puzzling, however it could indicate that the increase of CD4<sup>+</sup>CD25<sup>+</sup> cells in cord blood of fetuses from preeclamptic pregnancies, indicates the increased inflammatory state of this disease. This would be in line with other studies, reporting an increase in NK cells and CD8<sup>+</sup>CD25<sup>+</sup> cells in umbilical cord blood of neonates born to preeclamptic mothers (27,28).

The fetal immune system might be activated by maternal inflammatory mediators, which could have crossed the placenta. This could be a long-term influence on the immune system of offspring, therefore a follow-up will be performed on the offspring of the preeclamptic pregnancies.

In summary, our results demonstrate that preeclampsia is associated with a low percentage of circulating Treg cells in the mother. However, to imply a causal role, a decline in Treg cells before the onset of disease, needs to be investigated.

**Declaration of Interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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